Interactions between Coilin and PIASy partially link Cajal bodies to PML bodies

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Accepted 3 August 2005
Journal of Cell Science 118, 4995-5003 Published by The Company of Biologists 2005
doi:10.1242/jcs.02613

Summary

The eukaryotic nucleus contains a variety of dynamic structures, yet studies into the functional relationship of one type of subnuclear domain to another have been limited. For example, PML bodies and Cajal bodies associate, but the functional consequence of this association and the mediating factors are unknown. Here we report that an associated PML body/Cajal body can co-localize to an snRNA gene locus, with the Cajal body invariably situated between the PML body and the snRNA locus. Binding studies demonstrate that coilin (a Cajal body protein) directly interacts with PIASy (a PML body protein). Cell biological experiments using coilin and PIASy knockout cell lines demonstrate that interactions between coilin and PIASy account in part for the observed association of Cajal bodies with PML bodies. When the PIASy interaction region on coilin is deleted, the frequency of the association between Cajal bodies and PML bodies is reduced. These studies provide another example of coilin's role in the functional organization of the nucleus.

Key words: PIASy, SUMO, snRNPs, U snRNA gene loci, Nuclear organization

Introduction

The nucleus in eukaryotes is highly organized and contains numerous territories, domains and bodies (Lamond and Earnshaw, 1998; Spector, 2001; Zimber et al., 2004). The Cajal body and PML body are two such organelles. PML bodies are normally found in 10-20 nuclear foci and contain the promyelocytic leukemia (PML) protein plus numerous factors implicated in a variety of cellular functions such as transcriptional regulation (Matera, 1999a; Maul et al., 2000; Strudwick and Borden, 2002). Some classes of PML bodies have been shown to move throughout the nucleus in an ATP-dependent manner (Muratani et al., 2002; Wiesmeijer et al., 2002) and associate with transcriptionally active gene loci (Shiels et al., 2001; Wang et al., 2004). PML bodies also contain PIASy, which can serve as a SUMO (small ubiquitin-like modifier) E3 ligase (Sachdev et al., 2001). SUMO modification of transcription factors can lead to their retention in PML bodies and, thus, repress transcription (Girdwood et al., 2004).

Cajal bodies (CBs) are found in cells and tissues that are actively engaged in transcription, such as neuronal and cancer cells (Spector et al., 1992; Gall, 2000). Studies on *Xenopus* CBs reveal that they have a low-density, sponge-like structure that allows for macromolecules to exchange from the nucleoplasm to the CB (Handwerger et al., 2005). CBs contain the highest concentration of small nuclear ribonucleoproteins (snRNPs) in the nucleoplasm (Matera, 2003). Although snRNPs are necessary for pre-mRNA splicing, CBs do not play a direct role in the splicing process (Gall, 2000). Like PML bodies, CBs have been shown to move in an ATP-dependent manner (Platani et al., 2002; Platani et al., 2000) as well as associate with various gene loci (Frey and Matera, 2001). Some of the gene loci to which CBs associate transcribe the U snRNA components of the same snRNPs that are enriched within the CB. Consequently, CBs may provide a platform upon which a snRNP feedback regulatory mechanism may take place (Matera, 1999b).

Recent work has revealed the existence of small Cajal body specific RNAs (scaRNAs) that are localized exclusively in the Cajal body and are important for the posttranscriptional modification of U snRNAs; strengthening the role for CBs in snRNP biogenesis (Darzacq et al., 2002; Jady and Kiss, 2001). Most convincingly, work from the Kiss and Bertrand groups has shown that Sm snRNAs are indeed subject to modification (2′-*O*-methylation and pseudouridylation) in the Cajal body (Jady et al., 2003). These modifications are guided by scaRNAs and are important for the efficient splicing of pre-mRNA (Yu et al., 1998). Other work implicates CBs in U4/U6 assembly (Stanek and Neugebauer, 2004; Stanek et al., 2003), U4/U6.U5 tri-snRNP recycling (Schaffert et al., 2004), U2 snRNP biogenesis (Nesic et al., 2004) and U3 small nucleolar RNA transport (Boulon et al., 2004). Thus, CBs are important for several aspects of RNP formation (Ogg and Lamond, 2002; Carmo-Fonseca, 2002; Matera, 2003). Interestingly, there is often partial overlap between a PML body and a Cajal body in a given cell (Grande et al., 1996) but how and why this happens is unknown.

Various diseases are correlated with the disruption of PML and Cajal bodies or alteration in their protein composition (Spector, 2001; Zimber et al., 2004). For example, the marker protein for the PML body, PML, is fused to the retinoic acid receptor in acute promyelocytic leukemia, resulting in the

abolition of this structure (Kakizuka et al., 1991). Additionally, PML bodies are targeted by viral infections, presumably because the transcription factors present with these structures are needed for viral gene expression or replication (Maul et al., 2000; Moller and Schmitz, 2003). Cajal bodies display altered protein composition in patients with the neurodegenerative disorder spinal muscular atrophy (SMA). SMA is caused by mutations in a protein (SMN) that normally localizes to the cytoplasm and CBs. SMN is necessary for the cytoplasmic maturation steps of snRNP biogenesis (Meister et al., 2002). Because snRNPs are vital for splicing intronic sequences from pre-mRNA, it is suspected that the neuronal death observed in SMA is the result of inadequate RNA processing and subsequent diminution of the proteins necessary for cellular functions (Pellizzoni et al., 1998). Another neurodegenerative disorder, Machado-Joseph disease, alters the localization of both CBs and PML bodies (Yamada et al., 2001; Chai et al., 2002), but the functional consequence of this disruption is currently unknown.

The marker protein for CBs is considered to be coilin (Matera, 1999). Coilin has been found to interact with proteins vital for RNP biogenesis, such as Sm proteins of snRNPs and SMN (Hebert et al., 2001; Hebert et al., 2002). Coilin is important for proper CB formation and composition as evidenced by the fact that tissues and cell lines derived from coilin knockout mice do not have normal CBs but instead have 'residual' CBs that do not accumulate snRNPs or SMN (Tucker et al., 2001; Jady et al., 2003). Add-back experiments demonstrate that coilin can restore canonical CBs (Tucker et al., 2001). The proper targeting of snRNPs and snRNP biogenesis factors to the CB is Thus, contingent upon coilin. Coilin has also been shown to be important in the formation of gems, another subnuclear domain (Tucker et al., 2001; Hebert et al., 2001; Hebert et al., 2002; Boisvert et al., 2002). Gems (for Gemini of Cajal bodies) are found in a few cells lines and fetal tissues, and contain SMN and its associated Gemins (Liu and Dreyfuss, 1996; Young et al., 2001). Interestingly, in most cell lines and adult tissues, nuclear SMN is found within CBs. The presence of gems correlates with a decrease in the methylation of arginine residues within the C-terminus of coilin that directly interact with SMN (Hebert et al., 2002; Boisvert et al., 2002, Dundr et al., 2004). Coilin does not appear to be required for cellular viability, as some coilin knockout mice and cell lines derived from them have no blatant phenotypic alterations, apart from having residual CBs. However, there is a substantial decrease in the number of null mice born compared with what would be expected (Tucker et al., 2001). The observed decrease in viability may be the consequence of an inefficient RNA processing system due to a decrease in snRNP production.

Although it has been known for some time that CBs and PML bodies can associate with a high degree of statistical significance (Grande et al., 1996), the mechanism and functional consequence of this interaction has not been defined. To clarify how and for what purpose Cajal bodies and PML bodies associate, we initiated a series of investigations designed to reveal the factors responsible for this association. In this work, we demonstrate that direct interactions between the CB protein coilin and the PML body protein PIASy have a role in mediating the association of CBs to PML bodies. We also demonstrate that CBs and PML bodies can co-localize to the same U snRNA gene. These results further elucidate the role that coilin plays in the functional organization of the nucleus.

Materials and Methods

Yeast two-hybrid screen, plasmid construction and mutagenesis

A human brain cDNA library cloned into the prey vector pACT2 and pretransformed into the yeast strain Y187 (BD Biosciences, Palo Alto, CA) was mated with the strain PJ69-2A harboring the bait vector pAS2-1-coilin (aa 1-362, N362) per the manufacturer's instructions. After mating, the yeast were plated onto medium lacking tryptophan, leucine, histidine and adenine (to select for bait, prey and protein interaction). Colonies were picked after several days of incubation and the prey plasmid was isolated and transformed into PJ69-2A containing pAS2-1-N362 or control bait plasmids to confirm the specificity of the interaction. Restriction digests and sequencing revealed that PIASy was recovered multiple times in the screen. PIASy, coilin and coilin mutant constructs were made following standard molecular biology protocols or have been described (Hebert et al., 2000; Hebert et al., 2001; Hebert et al., 2002). For the directed two-hybrid screen between PIASy prey and various coilin baits, yeast containing the vectors were tested for the ability to grow on non-selective medium [lacking leucine and tryptophan (–LW), which selects for the bait and prey] or selective medium [lacking leucine, tryptophan, histidine and adenine (–LWHA), which selects for the bait, prey and protein interaction] after 3 days incubation.

Antibodies

The polyclonal antibody against GFP was purchased from BD Bioscience (Palo Alto, CA). The monoclonal GFP antibody and the digoxigenin antibody were from Roche (Mannheim, Germany). Other antibodies (myc and PML) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Fibrillarin was detected by using an antibody provided by Mark Olson (The University of Mississippi Medical Center). Coilin was detected using a polyclonal antibody made by Greg Matera or a monoclonal antibody developed by Maria Carmo-Fonseca (Almeida et al., 1998).

Cell culture, transfection, immunofluorescence and FISH

HeLa cells obtained from the American Type Culture Collection (ATCC) or wild-type and coilin knockout mouse embryonic fibroblast cells (MEF 26 and MEF 42) (Tucker et al., 2001) were cultured in DMEM media (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum in a 5% CO_2 incubator at 37°C, as were PIASy knockout mouse embryonic fibroblast cells (Wong et al., 2004). HeLa cells were transfected with Superfect (Qiagen, Valencia, CA) and processed as described (Hebert and Matera, 2000). MEF cells were transfected with Lipofectamine 2000 (Invitrogen) per the manufacture's instructions. Typically, cells growing in a one-well chamberslide (Nalge Nunc, Naperville, IL) were transfected with 4 μg of DNA and 10 μl of Lipofectamine 2000 mixed in 250 μl of Opti-MEM (Invitrogen) for 4-5 hours. The liposome complexes were then washed off with D-PBS and fresh culture medium was added to the chamberslide. The transfected cells were grown for 1-2 days before immunofluorescence. For immunofluorescence, cells grown on chamberslides were fixed with 4% paraformaldehyde followed by permeabilization with 0.5% Triton X-100. The permeable cells were then blocked with 10% normal goat serum for 30 minutes and probed with the corresponding primary and secondary antibodies for 30 minutes each. Cells were observed on a Nikon Eclipse E600 epifluorescent microscope. Fluorescent in situ hybridization (FISH) for U2 gene loci was carried out as described (Frey and Matera, 2001). All cell images were captured with the Coolsnap fx digital camera (Roper Scientific,

Tucson, AZ) and processed with MetaView (Universal Imaging Corporation, Downingtown, PA) and Adobe Photoshop 7.0 (San Jose, CA) software. When scoring cells for associations between nuclear bodies such as CBs, PML bodies and gems, an association was scored as positive if the two structures appeared to touch or be no more than half the diameter of a typical CB apart from each other, as observed with a 60× objective. A CB is roughly 0.5 μm in diameter.

Immunoprecipitation and in vitro binding assays

For co-immunoprecipitation experiments, the protocol detailed in Hebert and Matera (Hebert and Matera, 2000) was followed with a few modifications. Briefly, cells were lysed in a modified RIPA buffer [50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA plus 1 tablet of complete protease inhibitor cocktail (Roche, Mannheim, Germany) per 50 ml lysis solution]. The lysates were then repeatedly passed through a 25 gauge needle to shear DNA and subject to incubation with the appropriate antibodies for 1 hour at 4°C with gentle shaking. Following the addition of Protein A or G Sepharose (Amersham Pharmacia, Uppsala, Sweden) for 1 hour with gentle shaking, the beads were washed with 1 ml lysis solution five times. The beads were resuspended in 5× SDS loading buffer, boiled, and subject to SDS-PAGE and western blotting as described (Hebert and Matera, 2000). Input lanes typically account for 2% the amount of lysate used in the immunoprecipitation reactions. For in vitro binding assays, GST- and His-tagged constructs, after transformation into *E. coli* BL21(DE3)pLysS cells, were induced and purified as described (Hebert et al., 2001). In a binding reaction, approximately 1 μg of His-T7-tagged protein was incubated with 1 μg of the GST-fusion protein in 1 ml of mRIPA plus 2 mM DTT. After incubation for 1 hour at 4°C with gentle inversion, the beads were washed 5 times (1 ml each) with mRIPA plus DTT, resuspended in 15 μl 5× SDS loading buffer, boiled and subjected to SDS-PAGE. Primary antibodies used include anti-T7 (1:1000; Novagen, Madison, WI). Input lanes account for 1/5 the amount of protein used in the pulldown reactions.

Results

Cajal bodies and PML bodies can simultaneously associate with the same *RNU2* gene

To explore the cellular consequence of the association between CBs and PML bodies, we first determined the frequency of this association in HeLa cells. In agreement with previous results (Grande et al., 1996), we have found that 86% of fixed HeLa cells containing CBs (detected by anti-coilin staining) and PML bodies (detected by anti-PML staining) have at least one CB associated with a PML body. To investigate the interrelationship of Cajal bodies with PML bodies, we needed to establish if ectopically expressed FP-tagged coilin (a CB marker) and FP-tagged Sp100 (a PML body marker) (Muratani et al., 2002) would faithfully recapitulate the endogenous proteins and nuclear domains. Towards this end, we show that GFP-coilin is recruited to CBs that can associate with endogenous PML bodies in HeLa cells (Fig. 1, left panel). We next demonstrate that co-transfected CFP-coilin and YFP-Sp100 localize to nuclear bodies that can associate with each other (Fig. 1, right panel).

Since it is known that both CBs and PML bodies can associate with various gene loci (Matera, 1999a; Shiels et al., 2001; Wang et al., 2004), and PML bodies play a role in some aspect of transcriptional control (Strudwick and Borden, 2002; Shiels et al., 2001; Wang et al., 2004), we tested if CBs and

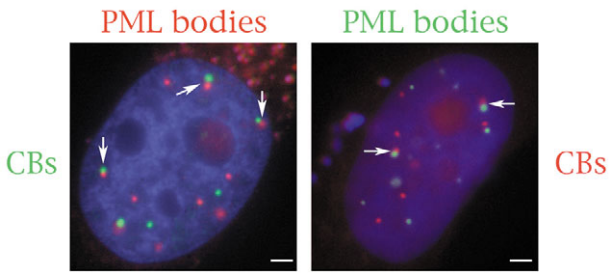

Fig. 1. Cajal bodies (CBs) can associate with PML bodies. (Left) HeLa cells were transfected with GFP-coilin to mark CBs (green) and PML bodies were detected with anti-PML antibodies (red). (Right) CBs and PML bodies formed by ectopically expressed proteins can associate. HeLa cells were co-transfected with CFP-coilin (red) and YFP-Sp100 (to mark PML bodies, green). In both panels, DAPI staining was used to identify the nucleus and associated CBs and PML bodies are marked with arrows. Scale bar, 2 μm.

PML bodies could co-localize to U snRNA gene loci. For this experiment, *RNU2* loci (which encode U2 snRNA) were detected by DNA FISH, while CBs and PML bodies were detected by anti-coilin and anti-PML immunostaining, respectively. As shown in Fig. 2, at least one CB is associated with an *RNU2* locus in approximately 65% of the cells examined. This frequency is in close agreement with previous reports (e.g. Frey and Matera, 1995). CBs and PML bodies can be observed next to the same *RNU2* locus in 36% of cells (n=49). The majority of cells (62%) did not have a PML body adjacent to an associated CB/*RNU2* locus. Only one cell scored had a PML body that was associated with an *RNU2* locus in the absence of a CB. Thus, CBs apparently do not require PML bodies to associate with U snRNA genes, but PML bodies are much more likely to be near U2 snRNA genes when associated

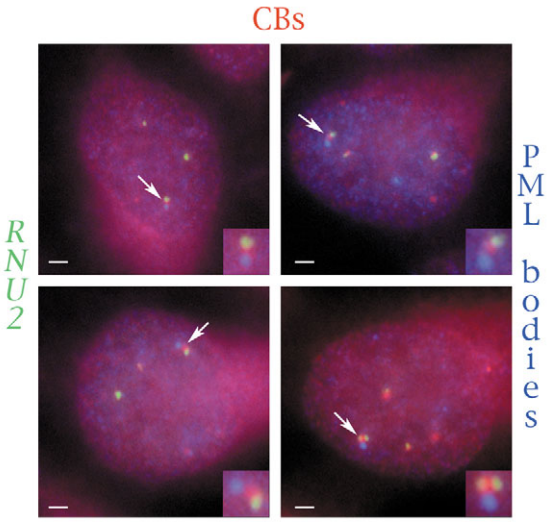

Fig. 2. Cajal bodies and PML bodies can associate with the same U2 gene locus. HeLa cells were subjected to antibody staining to mark the location of CBs (red) and PML bodies (blue), followed by DNA FISH using an U2 gene probe (green). The arrow marks an association between CBs, PML bodies and an U2 gene (inset) in four different cells. Scale bar, 2 μm.

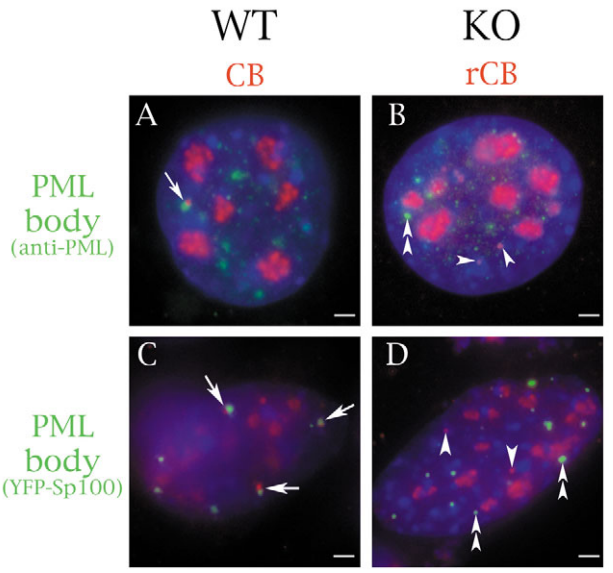

Fig. 3. Residual CBs containing fibrillarin do not associate with PML bodies. Wild-type (WT, panel A) and coilin knockout MEF cells (KO, panel B) were stained with antibodies to PML to mark PML bodies (green) and fibrillarin to detect CBs and residual CBs (red). Fibrillarin normally localizes to nucleoli and CBs, but is also enriched in one kind of rCB (Tucker et al., 2001). An arrow marks an association between a CB and a PML body (panel A), while arrowheads mark residual CBs in KO cells that fail to associate with PML bodies (double arrowhead, panel B). DAPI staining (blue) was used to define the nucleus. Wild-type (WT, panel C) and coilin knockout MEF cells (KO, panel D) were also transfected with YFP-Sp100 (green) to mark PML bodies and stained with anti-fibrillarin (red) to detect CBs and residual CBs (rCBs). Arrows mark some of the associations between CBs and PML bodies in wild-type cells (panel C). Double arrowheads mark PML bodies that fail to associate with rCBs (arrowheads) in knockout cells (panel D). Scale bar, 2 μm.

with CBs. Strikingly, when these three structures were associated, CBs were always positioned between the *RNU2* loci and PML bodies (Fig. 2, insets). The functional consequence of the PML/CB/*RNU2* triad, and its relevance to U2 snRNA gene expression is unknown.

Fibrillarin-containing residual Cajal bodies do not associate with PML bodies

We next set out to determine the factors that mediate CB association with PML bodies. Given the central role that coilin plays in the integrity of the CB, we reasoned that coilin, or an associated factor, is important for CB interaction with PML bodies. To test this, we utilized mouse embryonic fibroblast (MEF) cell lines that contain or lack coilin (Tucker et al., 2001). Coilin knockout MEF cells do not have typical CBs, but instead have two kinds of residual CBs. Residual CBs (rCBs) lack snRNPs and SMN but contain other antigens such as scaRNAs or Nopp140 and fibrillarin (Jady et al., 2003; Tucker et al., 2001). Normally, Nopp140 and fibrillarin localize to CBs and nucleoli. Thus, antibodies to fibrillarin can be used to detect canonical CBs and one kind of rCB. Wild-type MEF cells stained with anti-PML and anti-fibrillarin display an association between PML bodies and CBs in the majority of cells (84%, n=123, Fig. 3, panel A, arrow), consistent with our results from HeLa cells. By contrast, we never observed fibrillarin-containing rCBs in knockout MEF cells associated with PML bodies [n=61; Fig. 3, panel B, arrowheads (rCBs) and double arrowheads (PML bodies)]. Similar results were obtained when transfected YFP-Sp100 was used to mark PML bodies; wild type cells contained CBs that associated with Sp100 foci (panel C, arrows) but rCBs in knockout cells failed to associate with the Sp100 foci (panel D, arrowheads and double arrowheads). We also examined if PML bodies associate with gems in coilin knockout MEF cells and found that 93% of cells scored did not have an association between PML bodies and gems (n=128). These findings demonstrate that coilin, and not SMN, is necessary for CB association with PML bodies and suggest that coilin may be interacting with factors within the PML body to mediate this interrelationship.

Coilin directly interacts with PIASy, a protein enriched in PML bodies

To identify proteins in the PML body that may mediate the association with CBs, presumably via interactions with coilin, we conducted a yeast two-hybrid screen on a human brain cDNA library using an amino-terminal fragment of coilin as bait. Human coilin is 576 aa in length and residues in the C-terminus (from aa 362-576) have been shown to be important for direct interaction with SMN and the formation of gems, another subnuclear domain (Tucker et al., 2001; Hebert et al., 2001; Hebert et al., 2002; Boisvert et al., 2002). We speculated that the N-terminal portion of coilin (from aa 1-362) would also interact with proteins that play a role in CB function, so this region of coilin was used in the two-hybrid screen. The protein PIASy was recovered multiple times in the screen. This is a significant finding because PIASy has been shown to localize in PML bodies (Sachdev et al., 2001) and Thus, interactions between coilin and PIASy may link CBs to PML bodies. To delineate the interaction domain on coilin for PIASy, we tested for PIASy prey interaction with various coilin baits (Fig. 4A). Yeast containing both PIASy prey and full-length coilin (1-576 aa) bait are able to grow on selective medium, as are yeast that harbor PIAS prey and bait that contains the first 362 or 292 aa of coilin. By contrast, additional N-terminal coilin fragments (1-161, 1-92) and a C-terminal fragment (362-576) are unable to support growth. These experiments suggest that a fragment of coilin from aa 161 to aa 292 is important for association with PIASy.

To confirm this interaction and assess if it is direct, we conducted GST-pulldown assays on bacterially purified coilin and PIASy. Full-length coilin is recovered by GST-PIASy, but not by GST alone (Fig. 4B, lanes 5 and 4), proving that these two proteins interact directly. We also monitored PIASy interaction with N-terminal fragments of coilin. In agreement with the two-hybrid results, a fragment comprised of the N-terminal 362 aa of coilin is recovered by GST-PIASy (lane 9), but a C-terminal fragment from aa 362-576 (C214) is not (lane 13). Interestingly, in contradiction with the two-hybrid results, an N-terminal fragment of coilin comprised of the first 161 aa is recovered by GST-PIASy (lane 7). We speculate that the disparity in results between the two different protein interaction techniques with regard to coilin(1-161) binding to

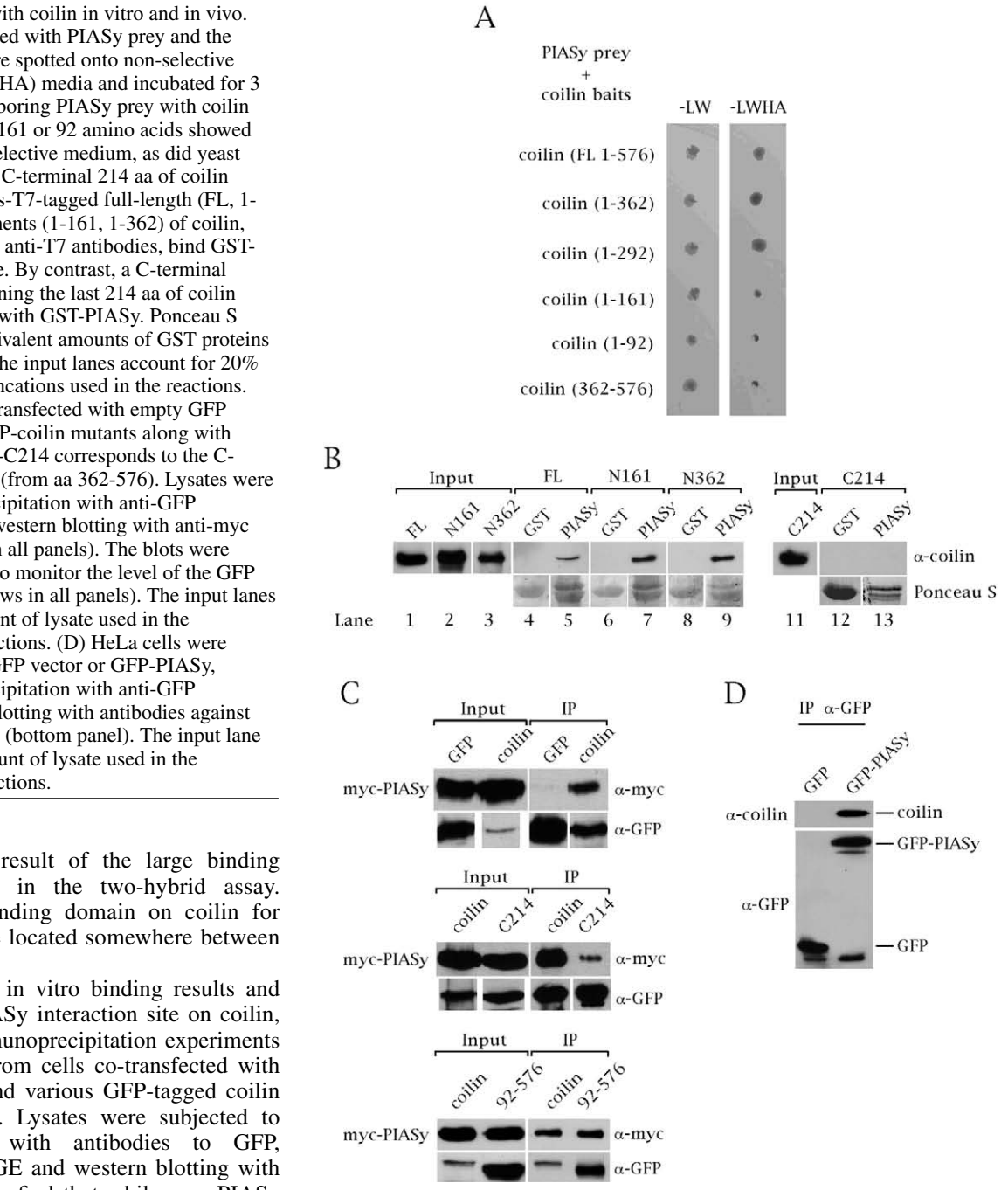

Fig. 4. PIASy interacts with coilin in vitro and in vivo. (A) Yeast cells transformed with PIASy prey and the indicated coilin baits were spotted onto non-selective (–LW) or selective (–LWHA) media and incubated for 3 days. Note that yeast harboring PIASy prey with coilin baits containing the first 161 or 92 amino acids showed minimal growth on the selective medium, as did yeast with PIASy prey and the C-terminal 214 aa of coilin (362-576) as bait. (B) His-T7-tagged full-length (FL, 1-576) or N-terminal fragments (1-161, 1-362) of coilin, detected by anti-coilin or anti-T7 antibodies, bind GST-PIASy but not GST alone. By contrast, a C-terminal fragment of coilin containing the last 214 aa of coilin (C214) does not interact with GST-PIASy. Ponceau S staining verified that equivalent amounts of GST proteins were used in the assay. The input lanes account for 20% of the coilin or coilin truncations used in the reactions. (C) HeLa cells were co-transfected with empty GFP vector, GFP-coilin or GFP-coilin mutants along with myc-tagged PIASy. GFP-C214 corresponds to the C-terminal 214 aa of coilin (from aa 362-576). Lysates were subjected to immunoprecipitation with anti-GFP antibodies, followed by western blotting with anti-myc antibodies (upper rows in all panels). The blots were reprobed with anti-GFP to monitor the level of the GFP tagged proteins (lower rows in all panels). The input lanes account for 2% the amount of lysate used in the immunoprecipitation reactions. (D) HeLa cells were transfected with empty GFP vector or GFP-PIASy, followed by immunoprecipitation with anti-GFP antibodies and western blotting with antibodies against coilin (top panel) or GFP (bottom panel). The input lane accounts for 2% the amount of lysate used in the immunoprecipitation reactions.

PIASy may be the result of the large binding domain fusion used in the two-hybrid assay. Consequently, the binding domain on coilin for PIASy may in fact be located somewhere between aa 92 and 292.

To corroborate the in vitro binding results and further define the PIASy interaction site on coilin, we conducted co-immunoprecipitation experiments using HeLa extract from cells co-transfected with myc-tagged PIASy and various GFP-tagged coilin or control constructs. Lysates were subjected to immunoprecipitation with antibodies to GFP, followed by SDS-PAGE and western blotting with antibodies to myc. We find that while myc-PIASy co-immunoprecipitates with coilin, little to no PIASy binds to GFP alone (Fig. 4C, upper panel). Reprobing of this same blot with anti-GFP demonstrates that an ample amount of GFP is precipitated, yet myc-PIASy is not recovered. To demonstrate that the N-terminal 292 aa of coilin are necessary for PIASy interaction, as suggested from the two-hybrid and GST-pulldown experiments, a GFP-tagged C-terminal coilin fragment consisting of aa 362-576 (C214) was tested for PIASy interaction. The amount of PIASy that binds to C214 is drastically reduced compared with that co-immunoprecipitating with full-length coilin (Fig. 4C, middle panel). These results, along with the GST-pulldown data, indicate that the N-terminal 292 residues of coilin play a major role in mediating the interaction with PIASy. To further delimit this region, we employed a coilin fragment that lacks the coilin self-association domain found within the first 92 residues of coilin (Hebert and Matera, 2000). This construct, coilin (92-576), is capable of binding myc-PIASy (Fig. 4C, lower panel). However, based upon the amount of immunoprecipitated coilin and coilin (92-576) detected by anti-GFP antibodies, the relative level of recovered PIASy is lower in the coilin (92-576) reaction compared with the coilin reaction. Therefore, PIASy probably binds between aa 92-292 of coilin, but the first 92 residues of coilin may indirectly facilitate this interaction by targeting coilin to the CB. We were unable to demonstrate if endogenous coilin and PIASy are present in a complex (our unpublished observations),

possibly reflecting that these proteins have a weak or transient interaction with each other. Such an interaction would be consistent with the dynamic nature of the association between CBs and PML bodies. Nevertheless, we can show that endogenous coilin can be cleanly co-immunoprecipitated with GFP-PIASy (Fig. 4D). Consequently, the abutment of CBs and PML bodies may be mediated by the interaction between coilin and PIASy.

Disruption of coilin/PIASy interaction reduces the frequency of Cajal body/PML body association

We have shown that coilin and PIASy interact both in cell extracts and directly in vitro. To further delimit the binding site on coilin for PIASy, and assess if mutations in coilin can alter the frequency of CB/PML body association, we conducted co-immunoprecipitation and cell biological assays using coilin knockout MEF cells. Coilin knockout MEF cells were co-

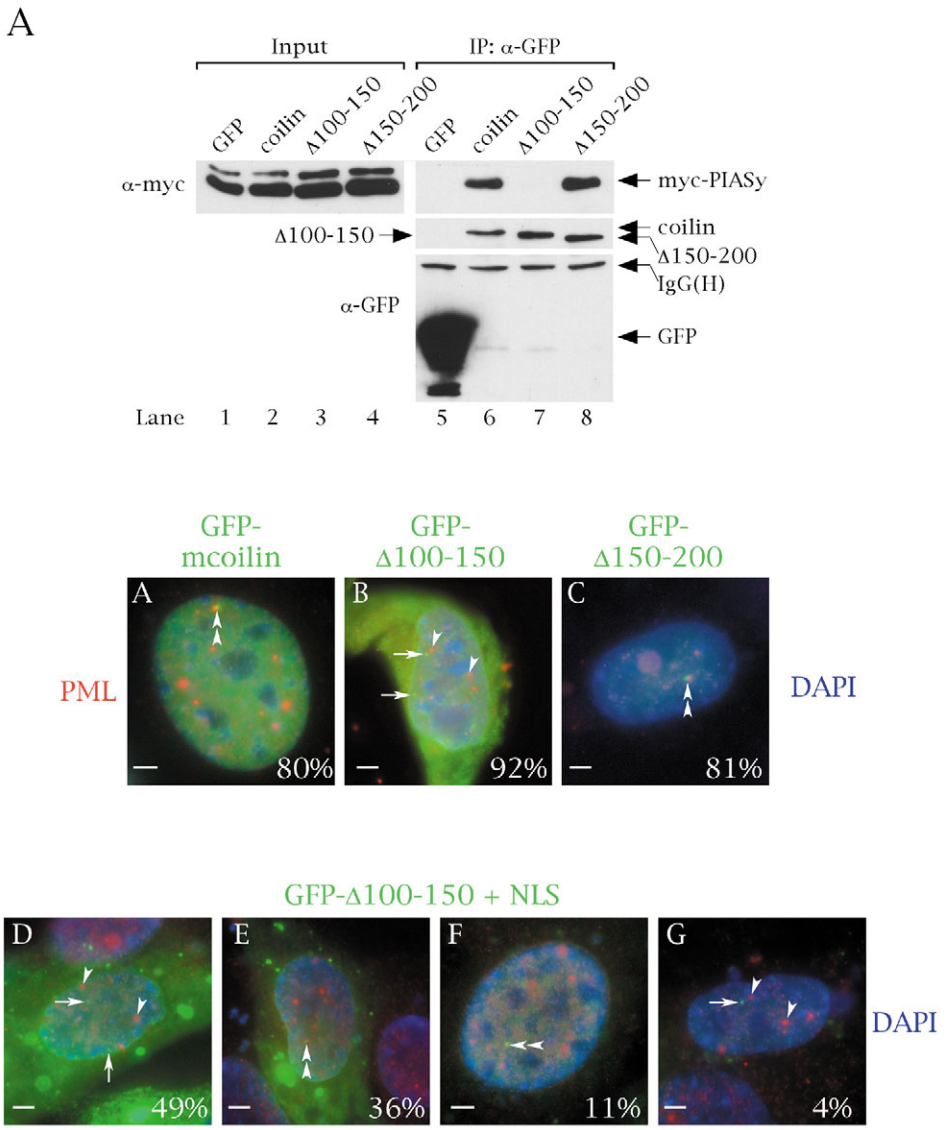

Fig. 5. A mutation in coilin that disrupts PIASy interaction reduces the frequency of CB/PML body association. (A) Coilin knockout MEF cells were co-transfected with myc-PIASy and various GFP-tagged control or mouse coilin constructs. After immunoprecipitation with anti-GFP antibodies and SDS-PAGE, a western blot was conducted with antibodies to the myc-tag (upper panels). The blot was reprobed with anti-GFP to verify that GFP, GFP-mouse coilin (coilin), GFP-mouse coilin (Δ100-150) and GFP-mouse coilin (Δ150-200) were immunoprecipitated (middle and lower panel). The position of the immunoglobulin heavy chain, IgG(H), is noted. (B) Coilin knockout MEF cells were transfected with full-length and mutant GFP-tagged mouse coilin constructs (green) followed by detection of PML bodies using anti-PML (red). DAPI staining (blue) was used to define the nucleus. Cells were scored for associations between CBs and PML bodies. In panels A-C, the percent in the bottom right of the image accounts for the majority of scored cells. For example, GFP-mouse coilin (panel A) forms CBs that associate with PML bodies (double arrowheads) in 80% of cells. By contrast, 92% of cells with CBs (arrows) formed by GFP-mouse coilin (Δ100-150) (panel B) do not show association with PML bodies (arrowheads). In panels D-G, coilin knockout MEF cells were transfected with GFP-mouse coilin (Δ100-150) + NLS and stained with anti-PML. The percent in the bottom right of each image accounts for the number of cells expressing this protein with similar localization patterns. For example, 49% of cells expressing GFP-mouse coilin (Δ100-150) + NLS (panel D) have cytoplasmic accumulations and CBs (arrows) which do not associate with PML bodies (arrowheads). By contrast, only 11% of transfected cells display a strictly nuclear coilin signal and CBs that associate with PML bodies (panel F, double arrowhead). Scale bar, 2 μm.

transfected with myc-tagged PIASy and various GFP-tagged mouse coilin or control constructs. Lysates were subjected to immunoprecipitation with antibodies to GFP, followed by SDS-PAGE and western blotting with antibodies to myc. As shown in Fig. 5A, full-length GFP-mouse coilin co-immunoprecipitates myc-PIASy, but GFP-alone does not (upper panel, compare lanes 5 and 6). Our previous results suggest that coilin residues between 92-292 are important for interaction with PIASy. To corroborate and extend these findings in a coilin knockout cell line, we generated two mutants of mouse coilin in which aa 100-150 or 150-200 are deleted. Co-immunoprecipitation experiments using coilin knockout cell extracts demonstrate that coilin mutant Δ100-150 does not form a complex with PIASy, but mutant Δ150-200 does (Fig. 5A, upper panel, compare lane 7 with 8). Reprobing of the same blot with anti-GFP antibodies shows that an equivalent level of GFP-mouse coilin or coilin mutants were recovered (middle panel) and a large amount of GFP-alone was precipitated (lower panel). Thus, coilin residues between 100-150 are important for interaction with PIASy.

If our hypothesis that interactions between coilin and PIASy link CBs to PML bodies is correct, then we should observe a reduction in the frequency of CB/PML body association in the presence of coilin mutant Δ100-150. For these experiments, coilin knockout cells were transfected with GFP-tagged full-length mouse coilin or mutants (Δ100-150 or Δ150-200), followed by scoring for CB/PML body association. Full-length mouse coilin forms CBs that associate with PML bodies in 80% of cells (n=81, Fig. 5B, panel A), in agreement with the results we found for HeLa cells and wild-type MEF cells. Coilin mutant Δ150-200, which interacts with PIASy, also forms CBs that associate with PML bodies in the majority of cells (81%, n=62, panel C). By contrast, coilin mutant Δ100-150 localizes primarily in the cytoplasm, presumably because it lacks one of two nuclear localization signals normally found within coilin (Gall, 2000). However some cells do contain CBs and the majority of these CBs (92%) fail to associate with PML bodies (n=28, panel B). Because the Δ100-150 mutation affects the localization of coilin in addition to altering coilin's ability to interact with PIASy, we engineered a nuclear localization signal (NLS) into the C-terminus of the GFP-tag on this construct. The majority (85%) of cells expressing GFP-mouse coilin Δ100-150 + NLS display cytoplasmic accumulations in addition to CBs, but little nucleoplasmic staining (n=53, panels D and E). Some cells (15%) expressing this construct have a localization pattern indistinguishable from wild-type coilin (panels F and G). Most of the observed cells (49%) had cytoplasmic staining with CBs that did not associate with PML bodies (panel D). Interestingly, CBs formed by Δ100-150 + NLS associate with PML bodies in only 47% of cells scored (panels E and F), compared with 80% association for full-length coilin. We have also verified in knockout cell extracts that mouse coilin (Δ100-150 + NLS) does not efficiently interact with PIASy (our unpublished observations). Thus, a mutation in coilin that inhibits PIASy interaction diminishes, but does not completely disrupt, the association of CBs with PML bodies. To further explore the role of the interaction between coilin and PIASy in mediating the interaction between CBs and PML bodies, we analyzed the association frequency of these nuclear subdomains in PIASy knockout MEF cells (Wong et al., 2004). Of the PIASy knockout cells scored, only 40% had CBs and PML bodies that were associated (n=265). This is significantly different than the association frequency observed in HeLa (86%) or wild-type MEF cells (84%), which contain PIASy. Moreover, this frequency (40%) is similar to that obtained in coilin knockout cells expressing coilin with a deletion in the PIASy interaction domain (47%). Therefore the lack of PIASy contributes to a reduction in the frequency of CB association with PML bodies.

Discussion

The work presented here provides mechanistic insight into how CBs associate with PML bodies. Our finding that coilin is necessary, in part, for the association of CBs with PML bodies provides another example of the significance of this protein in subnuclear organization (Fig. 6). Previous work has shown that coilin is vital for canonical CB number and formation, bringing together factors involved in RNP biogenesis into one subnuclear domain (Bauer and Gall, 1997; Hebert and Matera, 2000; Tucker et al., 2001; Shpargel et al., 2003; Jady et al., 2003). Additionally, dimethylation of arginine residues within coilin affects SMN interaction and gem formation (Tucker et al., 2001; Hebert et al., 2001; Hebert et al., 2002; Boisvert et al., 2002; Jady et al., 2003). Thus, coilin impacts CB and gem formation as well as PML body association.

Although CBs and PML bodies have been known to associate since 1996 (Grande et al., 1996), a systematic study to investigate how and why these domains associate has not been conducted. We find that coilin interaction with the PML body component PIASy accounts in part for the association of CBs with PML bodies. Given that PIASy is a SUMO E3 ligase, it is tempting to speculate that one consequence of this interaction is the modification of CB proteins by SUMO. We

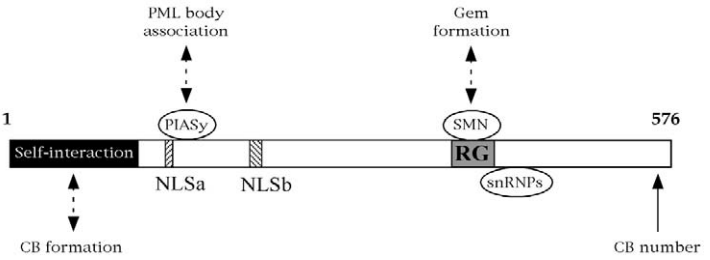

Fig. 6. Schematic summary of coilin and its role in nuclear organization. Human coilin is a protein of 576 aa and contains two nuclear localization signals (NLSa, NLSb) in addition to an arginine/glycine repeat region (RG). Previous work has shown that the N-terminal 92 aa of coilin are necessary for self-interaction and Thus, are important in CB formation (Hebert and Matera, 2000). Additionally, knockout studies demonstrate that full-length coilin is necessary for canonical CB formation (Tucker et al., 2001). The C-terminus of coilin is important in regulating CB number (Shpargel et al., 2003) and for the interaction with some Sm proteins and snRNPs (Hebert et al., 2001) (our unpublished observations). The RG box of coilin mediates direct interaction with SMN and reduction in arginine methylation correlates with Gem formation (Hebert et al., 2001; Hebert et al., 2002; Boisvert et al., 2002). The association of PML bodies with CBs is mediated in part by interactions between coilin and PIASy (this study). Not shown are interactions with Nopp140 (Isaac et al., 1998) and nucleic acids (Bellini and Gall, 1998).

have been unable to show that coilin is modified by SUMO in vivo or in vitro. However, it is possible that other proteins within the CB are subject to this modification during the CB/PML body association. Alternatively, interactions with coilin may regulate PIASy activity.

It is interesting that mutations in coilin that abolish PIASy interaction do not completely disrupt CB association with PML bodies. Likewise, PIASy knockout cells retain approximately half of the expected associations between CBs and PML bodies. We speculate that other proteins within the PML body may work in concert with PIASy to bridge these two structures. Such a protein could interact with a region of coilin separate from that which binds PIASy. Alternatively, an additional link between CBs and PML bodies may be mediated by a CB factor that interacts with coilin and PML body proteins. A detailed live-cell analysis of CB/PML body dynamics using wild-type and mutant coilin proteins should further clarify the mediating factors. Moreover, live-cell studies should also establish the longevity of the CB/PML body dialogue and determine if these nuclear bodies form at specific sites in the nucleus, such as U snRNA gene loci, or pair-up only at these locations.

To get an idea as to the functional consequence of the CB/PML body association on the regulation of *RNU2* expression, one has to consider what is known about why CBs and PML bodies associate with various genes. With regard to PML bodies, it is thought that their association with gene loci does not directly affect transcription, but instead allows for factors within the PML to be utilized by genes with high transcription demands. PML bodies, therefore, can serve as depots for factors required by dense transcription foci (Shiels et al., 2001; Negorev and Maul, 2001; Wang et al., 2004). Since Cajal bodies contain the snRNA-specific transcription factor, PTF-γ, CBs, like PML bodies, may also serve as reservoirs for factors needed for U snRNA expression (Schul et al., 1998; Gall, 2000). Alternatively, factors within the CB may downregulate the transcription of the gene to which it is associated (Matera, 1999a). Another possibility is that replication-dependent histone loci, to which CBs also associate (Gall, 2000), actually nucleate new CBs that, in turn, may be involved in basal nuclear RNA metabolism (Shopland et al., 2001). Since CBs require U2 snRNA for the association with *RNU2* loci (Frey and Matera, 2001) and pre-U2 RNA can be found within CBs (Smith and Lawrence, 2000), it is possible that CBs impact on many levels of U2 snRNA production and usage, from transcription to export to modification. Given the potential activities of CBs and PML bodies, an intriguing hypothesis is that the observed association of these two structures to the same gene locus may provide some type of regulatory control. Our finding that CBs are invariably positioned between *RNU2* loci and PML bodies, along with the observation that PML bodies only rarely associate with *RNU2* loci in the absence of CBs, suggests that the CB is the major regulatory subdomain and the PML body may provide additional transcription factors as needed. Disruption of the CB/PML body association may therefore be partially responsible for the observed viability problems in coilin knockout mice (Tucker et al., 2001), although similar problems have not been reported for PIASy knockout mice (Wong et al., 2004).

The YFP-Sp100 plasmid was a gift from David Spector (Cold Spring Harbor, NY). We thank Greg Matera (Case Western Reserve University) for the U2 gene plasmid, anti-coilin antibodies and the coilin knockout cell line. We also thank Rachel Kim and Hong Wu (HHMI, UCLA) for providing the PIASy knockout cell line and anti-PIASy antibody in an expeditious manner. This work was supported by an intramural research support program award from The University of Mississippi Medical Center, a grant from the Muscular Dystrophy Association (to M.D.H.) and the Luckyday Foundation (to S.H.S.).

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